REMARKS

Status of the Claims:

Claims 1-8 and 11-36 are pending and claims 1-8 and 11-20 are under consideration in this application. Claims 17-20 have herein been amended and support for these amendments can be found, for example, on page 8, lines 23-31. No new matter has been added. Claims 9, 10, and 21-36 have been cancelled without prejudice. All of the claims under consideration stand rejected.

35 U.S.C. 112, first paragraph, rejection

Claims 17-20 stand rejected on the grounds that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

From the comments at page 2, line 14, to page 4, line 3, of the Office Action, applicants understand the Examiner's position to be that, "the specification is merely speculative concerning success of an individual polypeptide as a diagnostic composition in methods for specifically detecting *M. tuberculosis* infected hosts or hosts susceptibility to *M. tuberculosis*" and thus the invention is not enabled by the specification in view of the state of the art at the time of filing.

Applicants respectfully traverse this rejection since there is ample evidence to support the contention that these polypeptides behave as claimed and defined by the specification.

The present invention is based on the discovery of a novel group of open reading frames (ORFs) encoding polypeptides that are secreted by *M. tuberculosis*, and provides for their use in diagnostic assays. As previously stated on page 13, line 18, to page 14, line 10, of the Amendment and Response of October 7, 2005, applicants submit that one of ordinary skill in the art would have expected a substantial number of the secreted *M. tuberculosis* polypeptides of the claimed methods to be useful in the diagnosis of tuberculosis. As further support for this

assertion, applicants provide a review article by A.S. Mustafa titled "Biotechnology in the Development of New Vaccines and Diagnostic Reagents Against Tuberculosis (A.S. Mustafa (2001) Current Pharm. Biotech. 2:157-173; herein referred to as Mustafa; and a copy of which is enclosed as Exhibit A). Mustafa discusses how secreted TB-antigens are highly immunogenic and were the subject of intensive investigation by artisans for this very reason. For example, on page 159, column 1, lines 26-30, Mustafa states "secreted antigens present in the culture filtrate of *M. tuberculosis* have attracted most attention because these are considered to be immunodominant and involved in protective immunity..." Moreover, a scientific article co-authored by one of the inventors of this application, Dr. Maria Laura Gennaro (Amor et al. (2005) Scandinavian J. Immunol. 61:139-146), confirmed that all five of the instantly claimed polypeptides tested were useful as diagnostics, either alone or in combination with a "prior art" polypeptide (see, for example, Amor et al., page 144, Fig. 4; and see, Office Action Response, October 7, 2005, page 13, line 23, to page 24, line 1). In Amor et al., each of the polypeptides specified by the claims (MTSP1, MTSP21, MTSP23, MTSP36, and MTSP43; or Rv0603, Rv1804c, Rv1271c, Rv2253, and Rv0203, respectively in Amor et al.), using an enzyme-linked immunosorbent assay (ELISA), were specifically recognized by antibodies in sera from a proportion of tuberculosis-infected patients. Moreover, sera from no control subjects, or from a much lower proportion of control subjects, contained such antibodies.

However, the Office action states on page 3, lines 6-12, that the Amor et al. reference was not found persuasive because: (i) "two of the claimed polypeptides MTSP1 (Rv0603) and MTSP43 (Rv0203) did not distinguish between a TB patient and a patient having lung disease other that TB...," and (ii) "[t]he remaining claimed polypeptides produced low reactivity with patient sera, i.e., at best 9/50 patients, at lowest 4/50 patients, above an arbitrary cutoff level." Applicants respectfully disagree with this position.

In response to the first point, applicants respectfully submit that the Amor et al. reference does show the two proteins of the claimed methods, Rv0603 and Rv0203, are antigenic/immunogenic and useful in TB serodiagnosis in patients. The authors' comments that "two of the claimed polypeptides MTSP1 (Rv0603) and MTSP43 (Rv0203) did not distinguish between a TB patient and a patient having lung disease other that TB" were in view of the raw data presented in Figure 3, which shows serological reactivity of the five novel secreted proteins

(of the instantly claimed methods) by comparison of patient (e.g., TB-positive subjects) and control populations. However, this in no way indicated that the two proteins lacked diagnostic potential or value. Quite the contrary, these proteins were believed to have and shown to have diagnostic utility as called-out first in the Abstract of Amor et al., page 139, lines 15-17, "thus at least five novel secreted proteins [Rv0203, Rv0603, Rv1271c, Rv1804c, and Rv2253] induce antibody responses during active disease," and demonstrated by the data presented in Figure 4 (see Amor et al., page 144).

Figure 4 of Amor et al. presents a re-analysis of the raw data of Figure 3 in which a cutoff value was set (i.e., + 3 standard deviations of ELISA readings from 48 non-TB control sera) – those patient test results which fell above the cutoff were determined to be "TB-positive" and those patient test results which fell below the cutoff were designated "TB-negative." Under these criteria, 8% (for Rv0203) and ~12% (for Rv0603) of TB-positive patients were sero-reactive to these two antigens, indicating that the two antigens are indeed useful in diagnostic tests for TB. Notably, using these criteria, the two polypeptides were not recognized by sera from any non-TB infected patients (control sera). Furthermore, one of the proteins, Rv0203, in combination with Rv1271 (MTSP23) further enhanced the predictive power of the p38 antigen (capturing 68% of TB-positive patients) in determining whether or not a patient was TB-positive (see, Figure 4, page 144, one column set from extreme right, and page 145, column 1, lines 10-11). Applicants respectfully point out that the analysis presented in Figure 4 of Amor et al is typical of how data from diagnostic tests are processed and provides more reliable diagnostic information than the raw data analysis presented in Figure 3.

In response to the second point raised by the Office Action, applicants respectfully submit that there is no requirement that the polypeptides used in the claimed methods perform better or even equivalently to those in the prior art. The only requirement is that the polypeptides of the claimed methods function as claimed and defined by the specification (see, for example, *Raytheon Co. v. Roper Corp. 724 F.2d 951, 958, 220 USPQ 592, 598 (Fed. Cir. 1983), cert. denied, 469 U.S. 835 (1984).* "An invention need not be the best or the only way to accomplish a certain result, and it need only be useful to some extent and in certain applications...[;]" *Cf. Custom Accessories, Inc. v. Jeffrey-Allan Industries, Inc., 807 F.2d 955, 960 n.12, 1 USPQ2d 1196, 1199 n.12 (Fed. Cir. 1986').* "It is possible for an invention to be less effective than

existing devices but nevertheless meet the statutory criteria for patentability[;]" or *E.I. du Pont De Nemours and Co. v. Berkley and Co., 620 F.2d 1247, 1260 n.17, 205 USPQ 1, 10 n.17 (8th Cir. 1980).* "The claimed invention must only be capable of performing some beneficial function... An invention does not lack utility merely because the particular embodiment disclosed in the patent lacks perfection or performs crudely....").

At page 3, lines 13-17, of the Office Action, the Examiner objects to the cited Amor et al. reference stating that "the method being claimed is not the method in the cited reference [Amor et al.]." Applicants respectfully point to M.P.E.P. 2164.02, "[c]ompliance with the enablement requirement of 35 U.S.C. 112, first paragraph, does not turn on whether an example is disclosed. An example may be 'working' or 'prophetic'....A prophetic example describes an embodiment of the invention based on predicted results rather than work actually conducted or results actually achieved." Indeed applicants respectfully submit that one skilled in the art would have believed that subjects whose sera showed reactivity in the ELISA assay of Amor et al. would very likely also have shown positive responses in the assay of claim 17, e.g., a skin test assay.

The Office Action also alleges at page 3, lines 19-20, that the "Amor et al. [reference] does not teach that the polypeptides can be utilized to diagnose a subject who is 'susceptible' to *M. tuberculosis*, as is claimed in the instant invention." While applicants respectively disagree with this position, to expedite the prosecution of this application, this term has been deleted from the first and second method steps of claims 17-20. In light of these amendments, applicants respectfully submit that this objection is moot.

From the comments on page 3, lines 17-18, of the Office Action, applicants understand Examiner's position to be that "functional segments" of a TB-specific polypeptides are not enabled by the specification. Applicants respectfully disagree with this position.

A functional segment of a TB-specific polypeptide, as defined in the specification, is "a segment of the polypeptide that has *Mycobacterium tuberculosis* specific antigenic and immunogenic properties." A polypeptide that has these properties, as defined by the specification (see page 7, line 18 to page 8, line 3), only need be recognized by and bind to antibodies elicited in response to *Mycobacterium tuberculosis* organisms or wild-type *Mycobacterium tuberculosis* molecules (e.g., polypeptides) and be able to elicit the production of antibodies that recognize and bind to Mycobacterium tuberculosis organisms or wild-type Mycobacterium tuberculosis

molecules (e.g., polypeptides). In view of: (a) this definition, or an even more rigorous definition in which the relevant antibodies bind to, for example, mycobacteria only of the *Mycobacterium tuberculosis* Complex; (b) the routine nature of testing for these antigenic and immunogenic properties; and (c) the small size of the these polypeptides, applicants submit that one of ordinary skill in the art would readily be able to identify segments of these polypeptides having the requisite function (although not necessarily the same level of function as corresponding full-length MSTP polypeptides). Moreover, doing so would not constitute undue experimentation.

In view of these considerations and amendments, applicants respectfully request that the rejection under 35 U.S.C. §112, first paragraph be withdrawn.

35 U.S.C. § 112, second paragraph, rejection

Claims 1-8 and 11-20 stand rejected as allegedly indefinite for failing to point out and distinctly claim the subject matter that the applicant regards as the invention. Applicants respectfully traverse this rejection.

At page 4, lines 18-20, the Office Action states "the specification does not provide guidance to which sequences and resulting polypeptides have the claimed M. tuberculosis specific antigenic and immunogenic properties."

As discussed above, a polypeptide having *Mycobacterium tuberculosis*-specific antigenic and immunogenic properties, as defined by the specification (see specification, page 7, lines 18-22; and page 7, line 30 to page 8, line 3), is one that can be recognized by and bind to antibodies elicited in response to Mycobacterium tuberculosis organisms or wild-type Mycobacterium tuberculosis molecules (e.g., polypeptides), and can elicit the production of antibodies that recognize and bind to Mycobacterium tuberculosis organisms or wild-type Mycobacterium tuberculosis molecules (e.g., polypeptides). This definition does not require that the relevant molecules elicit immune responses to molecules only expressed by the *Mycobacterium tuberculosis* Complex. In view of this definition, and especially in light of the teaching of the Mustafa et al. reference (see above), applicants respectfully submit that those skilled in the art would expect that most, if not all, of specified polypeptides would have *Mycobacterium tuberculosis*-specific antigenic and immunogenic properties.

Despite these considerations, and as shown by Table 2 of Amor et al. (page 142), all of the polypeptides specified by the instant claims are in fact specifically expressed by bacteria of the *Mycobacterium tuberculosis* Complex. The relevant polypeptides are MTSP15, 21, 25, 36, 43, and 47 (designated Rv0617, Rv1804c, Rv2398c, Rv2253, Rv0203, and Rv2290, respectively, in Amor et al.). In view of these findings, it is very likely that antibodies elicited by these polypeptides would not detect infections by mycobacteria other than those of the *Mycobacterium tuberculosis* Complex

In light of the above factors, applicants respectfully request that the Examiner withdraw the rejection under 35 U.S.C. § 112, second paragraph.

CONCLUSION

In summary, for the reasons set forth above, applicants maintain that the pending claims patentably define the invention. Applicants request that the Examiner reconsider the rejections as set forth in the Office Action and permit the claims to pass to allowance.

If the Examiner would like to discuss any of the issues raised in the Office Action, applicants' undersigned representative can be reached at the telephone number listed below.

Enclosed is a request for an automatic extension of time and a check in payment of the extension of time. Please apply any other charges or credits to deposit account 06-1050, referencing Attorney Docket No. 07763-042001.

Respectfully submitted,

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Biotechnology in the Development of New Vaccines and Diagnostic Reagents Against Tuberculosis

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Abstract: Tuberculosis (TB) is a disease of global concern. About one third of the world population is infected with *Mycobacterium tuberculosis*. Every year, approximately 8 million people get the disease and 2 million die of TB. The currently available vaccine against TB is the attenuated strain of *Mycobacterium bovis*, Bacillus Calmette Guerin (BCG), which has failed to provide consistent protection in different parts of the world. The commonly used diagnostic reagent for TB is the purified protein derivative (PPD) of *M. tuberculosis*, which is nonspecific because of the presence of antigens crossreactive with BCG and environmental mycobacteria. Thus there is a need to identify *M. tuberculosis* antigens as candidates for new protective vaccines and specific diagnostic reagents against TB. By using the techniques of recombinant DNA, synthetic peptides, antigen-specific antibodies and T cells etc., several major antigens of *M. tuberculosis* have been identified, e.g. heat shock protein (hsp)60, hsp70, Ag85, ESAT-6 and CFP10 etc. These antigens have shown promise as new candidate vaccines and/or diagnostic reagents against TB. In addition, recent comparisons of the genome sequence of *M. tuberculosis* with BCG and other mycobacteria have unraveled *M. tuberculosis* specific regions and genes. Expression and immunological evaluation of these regions and genes can potentially identify most of the antigens of *M. tuberculosis* important for developing new vaccines and specific diagnostic reagents against TB. Moreover, advances in identification of proper adjuvant and delivery systems can potentially overcome the problem of poor immunogenicity/short-lived immunity associated with protein and peptide based vaccines. In conclusion, the advances in biotechnology are contributing significantly in the process of developing new protective vaccines and diagnostic reagents against TB.

INTRODUCTION

In 1993, the World Health Organization (WHO) declared tuberculosis (TB) "a global emergency". The latest survey conducted by WHO showed that the problem of TB still exists at an alarming level with about one third of the world population infected and 8 million people developing the disease each year [1]. Moreover, among the existing 16.2 million cases of TB, 3.52 million patients (44%) suffered from infectious pulmonary disease. About 2 million people die of TB each year with global case fatality rate of 23%, which is above 50% in some African countries with high rates of human immunodeficiency virus (HIV) infection [1].

The global problem of tuberculosis is worsening due to several factors including the increase in incidence of multidrug-resistant (MDR) TB. The analysis of drug resistance in 35 countries showed that the prevalence of acquired resistance to any of the four first-line anti-TB drugs (isoniazid, rifampin, ethambutol, and streptomycin) ranged between 5.3% (in New Zealand) to 100% (in Ivanovo Oblast, Russia) with a median value of 36%. The combined prevalence of resistance to any of the four drugs tested ranged

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between 2.3% (in the Czech Republic) to 42.4% (in the Dominican Republic), with a median value of 12.6% [2].

The acquired immunodeficiency syndrome (AIDS) epidemic has further complicated the situation of TB. In 1997, the global prevalence of TB/HIV co-infection was estimated to be 0.18%, and 8% of the incident TB cases were infected with HIV [1]. In HIV-infected patients with TB, the immunodeficiency is associated with increased dissemination of TB, increased number and severity of symptoms, and rapid progression to death unless prompt and specific treatment is provided. The combination of MDR TB and HIV infection has a very bad prognosis with a median survival of only about two months [3].

The control and eventual eradication of TB requires an effective vaccine and reagents for specific diagnosis. The only available vaccine against TB is the bacillus Calmette Guerin (BCG), a live attenuated strain of virulent bovine tubercle bacillus *Mycobacterium bovis*. On the basis of promising results in animal models and >300 infants tested between 1921 and 1924, BCG vaccine was distributed around the world for the prevention of TB. Since then, BCG has been given to several billion people. Although, BCG is among the world's most widely used vaccine, and being directed against the world's leading cause of infectious disease mortality, it is the most controversial vaccine in current use. Estimates of protection imparted by BCG against pulmonary TB vary between nil to 80% (4). Moreover, the use of BCG vaccination faces two additional problems: i. BCG vaccination induces a delayed type hypersensitivity (DTH) response that cannot be distinguished from exposure to *M. tuberculosis*, and therefore it compromises the use of purified protein derivative (PPD) of *M. tuberculosis* in skin test for diagnostic or epidemiological purposes [5]. ii. BCG being a live vaccine is contraindicated in HIV infected individuals for fear of causing disease by itself.

With respect to diagnosis, PPD is routinely used as a skin test reagent for detection of *M. tuberculosis* infection. However, in all cases, a negative PPD test does not rule out a diagnosis of TB but may reflect the presence of anergy or incorrect administration of the test [6]. In addition, a positive PPD test may not distinguish between active disease, prior sensitization by contact with *M. tuberculosis*, BCG vaccination, or cross-sensitization by other mycobacterium species. Moreover, antigenic components in PPD are not standardized and therefore PPD from different sources may vary in the skin test response [7]. Thus there has been an urgent need to identify the antigens of *M. tuberculosis*, which could be candidates to develop improved vaccines with universal efficacy and specific diagnostic reagents for TB.

IDENTIFICATION OF *M. TUBERCULOSIS* ANTIGENS

The advances in biotechnology, in particular the use of DNA cloning and expression technologies, have greatly facilitated the identification of several major antigens of *M. tuberculosis*. However, the initial attempts to clone and express mycobacterial genes in other systems like *Escherichia coli* were met with a considerable degree of pessimism [8,9]. This was partly due to the belief that organisms such as mycobacteria with a high G + C content in their DNA must use radically different transcriptional control systems from those in *E. coli*, and that this effect would probably extend to translational signals as well, it would therefore be impossible to get useful levels of expression in *E. coli*. Later on, however, the problem was overcome when *E. coli* systems were successfully used to clone and express a large number of mycobacterial antigens. The first breakthrough in this field was reported by Young *et al.* who developed a lambda gt11 phage expression system for efficient expression of mycobacterial genes in *E. coli* [10,11]. Their system was based on the expression of antigens as beta-galactosidase fusion proteins, which are detected by immunoblotting of phage plaques on an *E. coli* lawn, and is ideally suited for proteins, which are recognized by antibodies. The fusion protein approach assures that the foreign sequence will be efficiently transcribed and translated in *E. coli*. In addition, fusion proteins are often more

resistant to proteolytic degradation than is the foreign polypeptide alone.

Screening of the mycobacterial recombinant DNA libraries with monoclonal and polyclonal antibody probes resulted in the identification and characterization of several mycobacterial antigens [10-12]. Further testing of the recombinant antigens expressed from specific recombinant phage clones with mycobacterial antigen reactive human T cell lines and clones showed that most of the recombinant antigens identified by antibody probes were also recognized by T cells [13-24]. Among these antigens, the antigens belonging to the family of heat shock proteins (hsp), i.e. hsp18, hsp60 and hsp70 were most frequently recognized by human T cells. However, the antigens recognized by antibodies may not always stimulate T cell responses, we therefore developed a system to directly screen T cell clones with the mycobacterial recombinant libraries to identify new antigens [25,26]. This approach led to the identification and characterization of a novel 24 kDa lipoprotein antigen with T cell epitopes shared between *M. tuberculosis* and *M. leprae*, but lacking in BCG and other mycobacteria [27].

In the recent years, secreted antigens present in the culture filtrate of *M. tuberculosis* have attracted most attention because these are considered to be immunodominant and involved in protective immunity [28]. It is suggested that secreted proteins are readily available for proteolytic processing and subsequent presentation by the infected host cell in the form of a peptide fragment associated with the major histo-compatibility complex (MHC) molecules. These surface-exposed fragments would allow the host immune system to recognize live pathogens sequestered within a host cell and to exert an antimicrobial effect against them. In particular, T cells may activate the host macrophage, allowing it to inhibit multiplication of intracellular organisms, or they may lyse the host cell, thereby denying the pathogens an intracellular milieu in which to multiply. Among the extracellular proteins of intracellular pathogens, the ones released in greatest abundance will be among the most effective in inducing immunoprotection. Such proteins, by virtue of their abundance in the phagosome, would be processed and presented most frequently, and therefore induce a particularly strong cell-mediated immune response [29].

To identify *M. tuberculosis* antigens actively secreted, and thus abundant in the culture filtrate, the antigens present in the short-term culture filtrate (ST-CF) were separated by biochemical techniques and tested for T cell reactivity with cells obtained from memory immune mice and TB patients. The results showed that T cells from the memory immune mice secreted large quantities of interferon-γ (IFN-γ) in response to two antigenic fractions. One of these fractions was represented by low molecular weight proteins (6 to 10 kDa proteins), and the other corresponded to the antigen 85 (Ag85) complex [30]. The individual antigenic components were further purified to obtain single proteins using biochemical fractionation and/or recombinant DNA technology. Testing of human T cells from tuberculosis patients showed that Ag85 and ESAT-6 were among the major stimulators of human T cells in proliferation and IFN-γ assays [31]. In the same assays, other secreted proteins, i.e. MPT64 and MPB70 were also found to be better stimulators of human T cells as compared to the antigens of cytosolic origin [32-34].

The Ag85 complex is among the most extensively studied secreted antigens of *M. tuberculosis*. It constitutes a family of fibronectin-binding proteins that are considered to be potential virulence factors. The three members of the family Ag85A, Ag85B, and Ag85C are encoded by the genes fbpA, fbpB, and fbpC, respectively. Members of the Ag85 complex are both secreted and retained in the cell wall of *M. tuberculosis* possess mycolyl transferase activity and could be involved in cell wall synthesis [35]. A clinical isolate of *M. tuberculosis* deficient in Ag85C was shown to contain 40% less cell wall-bound mycolates than the parent strain [36]. Furthermore, Ag85 complex is also required for the growth of *M. tuberculosis*, especially Ag85A. Loss of fbpA expression was shown to inhibit the ability of *M. tuberculosis* H37Rv to grow in wholly synthetic

media or to replicate in human or mouse macrophage-like cell lines, indicating that fbpA may play a role in pathogenesis of *M. tuberculosis* [37]. Ag85 complex proteins induce delayed hypersensitivity, protective immune responses, and specific antibodies in infected mice and guinea pigs [38-40]. Moreover, these proteins also induce cellular immune responses in cultured peripheral blood mononuclear cells of healthy PPD-positive people and TB patients with clinically active disease [41,42].

SYNTHETIC PEPTIDES TO IDENTIFY *M. TUBERCULOSIS* T CELL EPITOPES

The complete naturally purified or recombinantly produced antigens of mycobacteria, although less complex than the whole mycobacterial organisms are still immunologically quite complex. Most of these antigens may contain up to several hundred amino acids (aa), whereas the T cell epitopes usually range between 8 to 20 aa in length. Thus a complete antigen may have a large number of T cell epitopes; some of which could be beneficial and others harmful, e.g. the hsp60 has been shown to have epitopes capable of inducing T cells of helper [43] as well as suppressor types [44]. To exclusively identify the epitopes with protective potential, studies have been advanced to identify the epitopes of *M. tuberculosis* antigens recognized by human T cells of protective phenotype. In this direction, to identify the epitopes of major T cell antigens of *M. tuberculosis*, i.e. hsp18, hsp60, hsp70, ESAT-6, Ag85B, MPT64 and MPT70, peptides (18 to 25 mer, overlapping by 9 to 10 aa) covering the entire sequence of each protein were synthesized. These peptides were tested with peripheral blood mononuclear cells as well as human T cell lines and clones established against the whole cell *M. tuberculosis* and the complete antigens. The results showed that T cell epitopes were present in most of these antigens spanning the entire sequence (45-54). Thus no single peptide could replace the complete protein. However, in some proteins, dominant epitopes recognized by T cells from majority of the tested donors could be identified. It was of particular interest that all of the 8 peptides covering the sequence of ESAT-6 were stimulatory for T cells, however, the dominant T cell epitopes varied in patients of different geographical locations, most probably due to differences in the genetic background [45,54]. Thus, to be universally useful as a diagnostic reagent, a mixture of synthetic peptides would be required for the specific diagnosis of TB.

MHC RESTRICTION ANALYSIS OF *M. TUBERCULOSIS* ANTIGENS AND PEPTIDES

Human T cells recognize antigens/peptides in association with highly polymorphic human leukocyte antigen (HLA) class I and class II molecules. Mycobacterial antigens primarily activate CD4+ T cells, which recognize antigens/peptides mostly in association with HLA-DR molecules [55,56]. It is therefore essential that a potential vaccine or a diagnostic antigen to be useful in a population should be recognized by T cells in association with either the most frequently expressed and less polymorphic HLA-DR molecules like HLA-DR52 and HLA-DR53 [57] or multiple HLA-DR molecules in a promiscuous manner. To identify the HLA molecules restricting T cell recognition of the individual *M. tuberculosis* antigens and epitopes, T cell lines and clones responding to complete antigens and the respective peptides were tested for responses in the presence of defined anti HLA class I and class II antibodies. In most cases, only anti-HLA-DR antibodies inhibited the response (58-63). Thus suggesting that HLA-DR molecules are the main HLA molecules involved in the presentation of *M. tuberculosis* antigens to human T cells. When complete antigens were tested for HLA-restriction with a panel of HLA-DR typed antigen-presenting cells (APC), all of the tested complete antigens were presented to T cells by APC expressing varied HLA-DR types (Table **1**). Thus, several antigens fulfilled the criteria of promiscuous recognition, i.e. hsp18, hsp60, hsp70, 24 kDa antigen, ESAT-6 and Ag85B etc. (52-54, 58,59). In addition, some promiscuous T cell epitopes were identified on hsp18, hsp60, 24 kDa antigen, ESAT-6 and Ag85B by using synthetic peptides (Table **2**). These results suggested that at the complete antigen level, all of the tested antigens

Table 1. HLA Molecules Presenting the Major *M. tuberculosis* Antigens to T Cells

Antigen	Antigen presenting HLA-molecules
hsp18	All tested HLA-DR types (highly promiscuous)
hsp60	HLA-DR1, DR2, DR4, DR5, DR7, DR9, DR53
hsp70	HLA-DR1, DR2, DR4, DR5, DR7, DR9, DR53
24 kDa	HLA-DR53
ESAT-6	HLA-DR2, DR52, DQ2
Ag85B	All tested HLA-DR types (highly promiscuous)

fulfilled the HLA-requirement for inclusion as candidates for new vaccines. However, at the peptide level, a single autologous HLA-DR type presented most of the antigenic peptides, and only few peptides were promiscuously presented (52-54, 60-64). Such promiscuous peptides may be exploited in designing peptide-based vaccines and diagnostic reagents against tuberculosis.

CD1 RESTRICTED T CELL ANTIGENS OF *M. TUBERCULOSIS*

It has been known for a long time that T cells recognize peptide antigens in association with MHC molecules. However, nonpeptide antigens can be presented to T cells by non-MHC encoded CD1 molecules (65-67). CD1 molecules comprise a novel lineage of antigen-presenting molecules distinct from MHC class I and class II molecules. The CD1 molecules accommodate lipid and glycolipids antigens in their hydrophobic cavity for presentation to a wide variety of T cells, including CD8+ $\alpha\beta$ T cells and double negative $\alpha\beta$ and $\gamma\delta$ T cells (68, 69). Some CD1 molecules traffic to endosomal compartments for sampling mycobacteria-derived lipid antigens, and subsequently lipid antigen-loaded CD1 molecules are expressed on the cell surface to activate

Table 2. Peptides of *M. tuberculosis* Antigens Presented to T cell in Association with Frequently Expressed or Several HLA-DR Molecules

Peptide	HLA molecules involved in peptide presentation
hsp18 peptide (aa 41-55)	All tested HLA-DR types (highly promiscuous)
hsp60 peptide (aa 61-75)	HLA-DR1, DR2, DR7
hsp60 peptide (aa 141-155)	HLA-DR2, DR7, DR53
24 kDa (aa 207-214)	HLA-DR53
ESAT-6 (aa 72-95)	HLA-DR52, DQ2
Ag85B peptide (aa 10-27)	HLA-DR3, DR52, DR53
Ag85B peptide (aa 19-36)	All tested HLA-DR types (highly promiscuous)
Ag85B peptide (aa 91-108)	All tested HLA-DR types (highly promiscuous)

specific T cells (69). These CD1-restricted T cells kill mycobacteria-infected cells and secrete IFN-γ, suggesting a possible role of CD1-mediated T cell responses in immunity to mycobacterial infection (69,70).

IDENTIFICATION OF ANTIGENS SPECIFIC FOR *M. TUBERCULOSIS*

Most of the studies described above identified antigens that are shared between *M. tuberculosis*, BCG and environmental mycobacteria. Although, potentially useful in vaccine design, these antigens may not be useful in specific diagnosis of tuberculosis. Therefore, several research groups are now focusing the work to identify antigens specific for *M. tuberculosis*. Moreover, it is difficult to predict how the crossreactive antigens will fare as vaccines in humans because among the hypotheses to explain the failure of BCG vaccines against tuberculosis is the exposure of the vaccinated population to environmental mycobacteria [4]. In addition, the presence of crossreactive antigens reduces the utility of PPD in the diagnosis of tuberculosis in BCG vaccinated healthy individuals. It is therefore considered that the antigens specific for *M. tuberculosis* and absent in BCG and environmental mycobacteria may provide reagents for specific diagnosis and vaccines with consistent protective efficacy.

By suing techniques of biotechnology, Mahairas *et al.* [71] opened a new field of study to identify *M. tuberculosis* specific antigens. They employed subtractive genomic hybridization analysis to identify genetic differences between virulent *M. bovis* and *M. tuberculosis* and attenuated/avirulent BCG. Three distinct genomic regions of difference, designated RD1, RD2 and RD3, were found deleted in BCG [71]. RD3 is present in virulent laboratory strains of *M. bovis* and *M. tuberculosis* and is absent from BCG and 84% of virulent clinical isolates, and therefore the antigens expressed from this region will not be of much interest as vaccines or diagnostic reagents against TB. RD2 is conserved in all virulent laboratory and clinical tubercle bacilli tested and was deleted only from substrains derived from the original BCG Pasteur strain after 1925. Thus the antigens encoded by the RD2 region may be useful as vaccine candidates and diagnostic reagents in areas where BCG strains lacking RD2 are used to vaccinate people against TB. MPT64, a 23kD secreted protein encoded by a gene in the RD2 region, elicits T-cell responses and cutaneous delayed type hypersensitivity (DTH) reactions in *M. tuberculosis* infected animals [72]. Patients with tuberculosis and their tuberculin-positive contacts respond to the antigen MPT64, but recipients of BCG vaccine strains lacking the mpt64 gene do not. Moreover, animals sensitized with BCG strains lacking the mpb64 gene (the homologue of mpt64 gene in *M. bovis*) don't respond to MPT64 [73]. The mpt64 gene is present in *M. tuberculosis* substrains H37Rv, H37Ra, and the Erdman and in the *M. bovis* BCG substrains Tokyo, Moreau, and Russian, whereas the *M. bovis* BCG substrains Glaxo, Pasteur, Canadian, Tice, and Danish 1331 and *M. leprae* lack the mpt64 gene [74]. This is consistent with the fact that RD2 is absent from BCG substrains derived from the original BCG Pasteur strains after 1925 [71]. MPT64 has been characterized as a powerful skin test antigen which can distinguish guinea pigs immunized with *M. tuberculosis* from guinea pigs immunized with other mycobacteria, i.e. BCG Danish 1331 and *Mycobacterium avium* [75].

RD1, a 9.5kb DNA segment found to be deleted from all BCG substrains is conserved in all of the tested virulent laboratory and clinical isolates of *M. bovis* and *M. tuberculosis*. It is therefore considered to be the most important *M. tuberculosis* specific region described by Mahairas *et al.* for the development of new vaccines and reagents for specific diagnosis of TB. The reintroduction of RD1 into BCG resulted in a protein expression profile almost identical to that of virulent *M. bovis* and *M. tuberculosis* [71]. In their original publication, Mahairas *et al.* annotated eight potential ORFs (ORF1A to ORF1K) in the RD1 region (Table **3**). In the genome sequence of *M. tuberculosis* H37Rv, Cole *et al.* annotated 11 ORFs (Rv3870 to Rv3880) in this region, 7 of which matched with those identified by Mahairas *et al.* (Table **3**). In our study, the RD1 sequence was analyzed for ORFs by using GeneMark software. This software

Table 3. Description of *M. tuberculosis* RD1 Region ORFs

ORF Code	Mahairas annotation	Cole annotation	Position on RD1	Length of protein no. of aa	Status in BCG
ORF1		Rv3870	1-786	261	Present
ORF2	ORF1A	Rv3871	889-2664	591	Deleted
ORF3		Rv3872	2807-3103	98	Deleted
ORF4			4014-3595	239	Deleted
ORF5	ORF1B	Rv3873	3128-4243	371	Deleted
ORF6		Rv3874	4336-4638	100	Deleted
ORF7	ORF1C	Rv3875	4671-4958	95	Deleted
ORF8			5502-5082	139	Deleted
ORF9	ORF1D	Rv3876	5072-7072	666	Deleted
ORF10	ORF1E	Rv3877	6946-8604	552	Deleted
ORF11	ORF1F	Rv3878	8755-9597	280	Deleted
ORF12	ORF1G		9787-11478	563	Deleted
ORF13	ORF1K	Rv3879	11895-9655	746	Deleted
ORF14			10577-11365	262	Deleted
ORF15			11731-12018	95	Deleted
ORF16		Rv3880	12659-12312	115	Present
ORF17			14020-12656	460	Present
ORF18			15533-14145	462	Present
ORF19			16900-15530	456	Present
ORF20			14799-17092	135	Present

identifies ORFs based on initiation and stop codons, comparison of ORFs sequences to sequences in the database using probabilistic statistics, and analysis of the ribosomal sites' sequence. The analysis resulted into the identification of a total of 20 potential ORFs (ORF1 to ORF20) in the RD1 region of *M. tuberculosis* (Table 3). Their organization and the predicted orientation of expression are shown in (Fig. 1). The putative proteins expressed from 14 ORFs, i.e. ORF2 to ORF15 are specific for *M. tuberculosis/M bovis*, whereas the proteins expressed from the remaining six ORFs of RD1, i.e. ORF1 and ORF16 to ORF20, are also present in BCG (Table 3). The proteins expressed from *M. tuberculosis/M. bovis*-specific ORFs of RD1 region could be involved in pathogenesis, useful in specific diagnosis and development of new

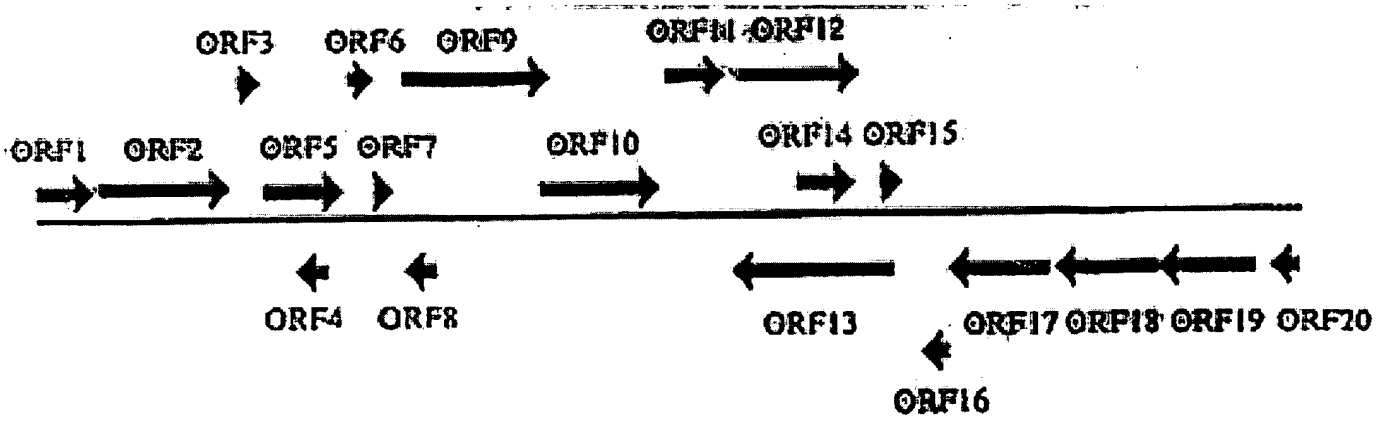

Fig. (1). RD1 region ORFs and orientation of their expression.

vaccines against TB. The immunological value of this region could be predicted because ORF7 of RD1 encodes ESAT-6, a major T cell antigen of *M. tuberculosis* (31). Thus it was of interest to determine if other ORFs of RD1 region could also have immunological reactivity.

To functionally characterize the putative proteins encoded by the ORFs of RD1 region, we first attempted to clone and express six of them (ORF10 to ORF15) as recombinant proteins in *E. coli*. However, we were successful in expressing five and purifying only two of the six targeted proteins [76]. One of these, i.e. ORF14 protein was frequently recognized by antibodies in the sera of tuberculosis patients but not healthy BCG vaccinated donors [76]. To analyze further all of the *M. tuberculosis/M. bovis* specific RD1 proteins for expression and immunological reactivity, a novel approach of overlapping synthetic peptides was used. The peptides were tested for reactivity with peripheral blood mononuclear cells and T cell lines in proliferation and IFN-γ assays. This analysis showed that, in addition to ESAT-6, the RD1 region contains other dominant antigens stimulatory for T cells of protective phenotype [77].

Among all the *M. tuberculosis* specific RD1 region gene products, the ESAT-6 (encoded by ORF7) is the most well characterized protein. The gene for ESAT-6, an early secreted antigen of *M. tuberculosis*, is deleted from all the BCG substrains [78]. Native and recombinant ESAT-6 are immunologically active to elicit a high level of IFN-γ from memory-immune mice challenged with *M. tuberculosis*. Analyses of subcellular fractions of *M. tuberculosis* showed the presence of ESAT-6 in cytosolic- and cell wall-containing fractions as well [79]. Several studies have shown that ESAT-6 is a major antigen of *M. tuberculosis* inducing protective Th1-type immune response in animals as well as in humans [80-85]. Cellular immune responses to ESAT-6 discriminate between patients with pulmonary disease due to *M. tuberculosis* and *M. avium* [86], and thus this antigen may have potential in specific diagnosis of TB.

CFP10, a 10 kDa protein present in ST-CF of *M. tuberculosis* and encoded by ORF6 of RD1 region, was first identified by Berthet *et al.* who studied the promoter region of the *esat-6* gene and found another cotranscribed gene *1hp* coding for a low molecular mass protein [87]. Similarly, Skjot *et al.* have described another low-mass *M. tuberculosis* protein, TB10.4, belonging to the ESAT-6 family and having 40% homology with the *esat-6* gene [88]. The three members of the ESAT-6 family (ESAT-6, CFP10, and TB10.4) share striking immunodominance in the human immune response against *M. tuberculosis* and induce high levels of INF-γ [88]. Members of this family have shown promising results to improve the specific diagnosis of TB by using serological as well as cellular methods for pulmonary and extrapulmonary disease [89-93]. Furthermore, overlapping peptide pools covering the entire sequence of ESAT-6 and CFP10 are as effective in T cell responses as the respective full-length

proteins, and thus the mixtures of synthetic peptides could replace the need of full-length proteins in the specific diagnosis of TB [94,95].

In another major attempt to identify functional proteins of *M. tuberculosis*, Arruda *et al.* identified a DNA fragment of *M. tuberculosis*, which when introduced into *E. coli*, enabled the transformed bacteria to enter and survive in the HeLa cell lines and macrophages [96]. The protein encoded by the DNA fragment was designated mammalian cell entry protein (Mce). The complete genome sequence of *M. tuberculosis* has shown that the Mce protein is part of an operon that is capable of encoding eight proteins [97]. Six of these proteins are specific for mycobacteria. In addition there are four homologous *mce* operons (*mce* 1-4) dispersed in *M. tuberculosis* genome and organized in exactly the same way. Each *mce* operon is capable of encoding six mycobacteria-specific proteins MceA to MceF (Table **4**). The available sequence from *M. leprae* genome has confirmed the existence of an operon homologous to *mce1* (Table **4**). Our studies have shown that all of the six Mce proteins encoded by *mce1* operon are expressed in *M. tuberculosis* and immunologically active in animal models [98]. In addition, cloning and expression of Mce1A to Mce1E in *E. coli* and screening with sera from tuberculosis patients have revealed that these proteins are immunologically active in humans as well [99]. However, from diagnostic point of view the proteins encoded by *mce3* operon may be more important because this region is specific for *M. tuberculosis* (Table **4**).

To identify additional *M. tuberculosis* specific regions and genes, a detailed comparative genome analysis by using DNA-microarray technology has been done by Behr *et al.* [100]. The analysis was performed for differences between *M. tuberculosis*, *M. bovis*, and the various BCG strains. The results showed that a total of 16 regions present in *M. tuberculosis* H37Rv (RD1 to RD16) were deleted in some or all strains of *M. bovis* and/or BCG (Table **5**). Among these, 11 regions (RD3 to RD7, RD9 to RD13 and RD15) covering 91 ORFs of *M. tuberculosis* H37Rv were deleted from one or more virulent strains of *M. bovis*. Five additional regions (RD1, RD2, RD8, RD14 and RD16) representing 38 ORFs were present in *M. bovis* but absent from some or all BCG strains (Table **5**). The deleted regions include the genomic segments covered by RD1 and *mce3* operon (RD15) described previously (71,97). Cloning and expression of these *M. tuberculosis*-specific RD regions followed by testing for immunological reactivity may identify additional antigens with potentials in specific diagnosis and new vaccines with universal efficacy to protect against TB.

NEW VACCINES

A number of *M. tuberculosis* antigens recognized by Th1 cells of protective phenotype can be considered promising as new vaccines to protect against TB in humans. These include protein as well as non-protein antigens. The most widely studied antigens to develop new vaccines against TB are the protein antigens hsp65, Ag85 and ESAT-6. A number of approaches including recombinant antigens as subunit vaccines or genes of the respective antigens cloned in appropriate plasmid vectors in the form of DNA vaccines have

Table 4. Mce encoding Operons of *M. tuberculosis* and their Distribution in other Mycobacteria

Operon	Total no. of ORFs	No. of mycobacteria specific ORFs	Presence in mycobacterial species
mce1	8	6	*M. tuberculosis, M. leprae, M .bovis*
mce2	8	6	*M. tuberculosis, M. bovis*
mce3	8	6	*M. tuberculosis*
mce4	8	6	*M. tuberculosis, M. bovis*

Table 5. RD Regions of *M. tuberculosis* H37Rv Deleted in *M. bovis* and BCG

Deletion region	Deletion in		No. of ORFs	ORFs deleted
	M. bovis Strains	BCG strains		
RD1	Not deleted	All strains	9	Rv3871-Rv3879c
RD2	Not deleted	Danish, Prague, Glaxo, Frappier, Connaught, Phipps, Tice, Pasteur	11	Rv1978-Rv1988
RD3	3/8	All strains	14	Rv1573-Rv1586c
RD4	8/8	All strains	3	Rv0221-Rv0223c
RD5	8/8	All strains	5	Rv3117-Rv3121
RD6	8/8	All strains	11	Rv1506c-Rv1516c
RD7	8/8	All strains	8	Rv2346c-Rv2353c
RD8	Not deleted	Frappier, Connaught	4	Rv0309-Rv0312
RD9	8/8	All strains	7	Rv3617-Rv3623
RD10	8/8	All strains	3	Rv1255c-Rv1257c
RD11	8/8	All strains	5	Rv3425-Rv3429
RD12	8/8	All strains	4	Rv2072c-Rv2075c
RD13	4/8	All strains	16	Rv2645-Rv2660c
RD14	Not deleted	Pasteur	8	Rv1766-Rv1773c
RD15	8/8	All strains	15	Rv1963c-Rv1977
RD16	Not deleted	Moreau	6	Rv3400-Rv3405

been attempted to protect against TB in animal models. As given below, both of these approaches have been successful in protecting against challenge with live *M. tuberculosis* in mouse and guinea-pig models of TB, as shown by increased survival, reduced pathology and decrease in number of bacilli recovered from organs of vaccinated animals as compared to non-vaccinated or sham-vaccinated controls. However, none of these candidate vaccines have yet been evaluated for safety in humans. Moreover, before routine application of any of the candidate vaccines for protection against TB in humans, these must be evaluated for efficacy in control trials conducted on large scales and in different parts of the world. An ideal vaccine should provide long-term protection and effectiveness in both immuno-competent as well as immuno-compromised individuals, irrespective of the geographical location of use.

SUBUNIT VACCINES

Subunit vaccines based on recombinant antigens/peptides, although safe for immuno-competent as well as immunodeficient individuals, require an efficient delivery system to induce a

strong and long lasting protective immunity. This requirement may be overcome by using an appropriate adjuvant. A subunit vaccine, consisting of a few key molecules of *M. tuberculosis*, which are capable of inducing protective immunity, could have advantages over BCG or other whole bacterium vaccines. Since the subunit vaccine would consist of only a few selected molecules rather than the thousands of molecules that make up a whole bacterium, it is more likely to be safe. Moreover, a subunit vaccine can be constructed so as to eliminate irrelevant or even immunosuppressive components of the whole bacterium, and therefore it might induce a stronger protective immune response than a whole-bacterium vaccine. Finally, in contrast to BCG, the subunit vaccine can be rigorously standardized, allowing the production and use of a consistent preparation and hence meaningful predictions can be made regarding its efficacy and safety.

Several studies have shown that non-living subunit vaccines, mostly including secreted proteins from the culture filtrate (CF) of *M. tuberculosis*, provide protective immunity in mice and guinea pigs models of TB [101-103]. When mice were immunized with ST-CF of actively growing *M. tuberculosis* with the surface-active agent dimethyldioctadecylammonium chloride as an adjuvant, a dose-dependent vaccine specific Th1-type immunity was induced in the animals, which efficiently protected the animals against a subsequent challenge with virulent *M. tuberculosis* [101]. The ST-CF vaccine induced a wider T-cell reactivity, directed to multiple secreted antigens, than did the viable BCG vaccine that induced a restricted T-cell reactivity directed only to two secreted protein fractions with molecular masses of 5-12 kDa and 25-35 kDa [101]. The study further showed that the ST-CF vaccine induced a population of long-lived CD4+ T cells, which could be isolated 22 weeks after the vaccination and could adoptively transfer acquired resistance when injected into T-cell-deficient mice [101]. This study demonstrated, for the first time, the potential of the subunit vaccine in replacing live recombinant vaccines for inducing protective immunity, in addition to their edge of being safe even in immunocompromised subjects.

In another study using guinea pig model of TB, Horwitz *et al.* further showed the potentials of purified major extracellular proteins of *M. tuberculosis* as components of a subunit vaccine. The immunized animals were protected against weight loss, death, and growth of *M. tuberculosis* in their lungs and spleens [102]. Again using the guinea pig model, Baldwin *et al.* compared the efficacy of two vaccine formulations, one based on *M. tuberculosis* culture filtrate along with adjuvant, and the other based upon a DNA plasmid vaccine encoding the culture filtrate Ag85A of *M. tuberculosis* [102]. Both of the vaccines induced lymphocytic granulomatous response, similar to that seen in animals vaccinated with BCG. The new vaccines, however, could prolong the survival of infected guinea pigs and prevented them from developing caseating disease. Neither of the non-living vaccines induced skin test reactivity to commercial tuberculin which added value to these approaches in constructing vaccine against TB. The only drawback reported to the non-living vaccines in this study was that the survival of vaccinated guinea pigs was shorter than that conferred by BCG. In spite of this drawback, Baldwin *et al.* considered this new approach in vaccine development as promising and suggested that the new vaccine formulations could be effectively used to boost protective immunity in individuals who have been previously vaccinated with BCG or those who may be at risk of reactivation disease due to latent TB, or drug-resistant TB that is refractory to treatment with the available anti-TB drugs [103].

With respect to studies with a single antigen, it has been shown that immunization with ESAT-6 when given along with an appropriate adjuvant combination provided protection in mice. The level of protection was comparable to that achieved after vaccination with live BCG [104]. However, immunization with ESAT-6 did not provide protection when the antigen was given alone or in the absence of appropriate adjuvant combination [104]. Such protection studies were further extended to determine the efficacy of

peptide based vaccines by using single peptides from ESAT-6 sequence as immunogens [105]. The peptides were emulsified with dimethyl dioctadecylammonium bromide/monophosphoryl lipid A/IL-2 and tested as experimental vaccines in i.v. and aerosol model of tuberculosis in mice. The immunization induced high level of cellular immune response and the epitope 51-70 of ESAT-6 induced significant level of protective immunity. Interestingly the level of protection was equivalent to that achieved with the complete ESAT-6 protein and BCG [105]. These results demonstrate the potential of peptide-based vaccines in protection against TB.

DNA VACCINES

Much progress has been made to use naked plasmid DNA as a system to deliver *M. tuberculosis* antigens because DNA based vaccines have inherent adjuvant activity, do not have the problem of pathogenecity associated with live recombinant vaccines and induce long lasting immunity. In addition, a single immunization with DNA vaccines may be sufficient to induce a strong protective immune response, thus avoiding repeated immunization that is required for subunit vaccines in adjuvant combinations. DNA delivery systems may also be exploited to deliver multiple antigens by cloning several genes in the desired plasmid DNA [106]. Several trials with DNA vaccination have shown protection of mice against subsequent challenge with *M. tuberculosis* by establishing a cellular immune response dominated by antigen-specific T lymphocytes that produce INF-γ and are cytotoxic towards infected cells [107-111]. A candidate for this type of vaccine, which has been extensively studied, is Ag85 of *M. tuberculosis* [110, 111]. Mice intramuscularly injected with plasmid DNA encoding the three components of the immunodominant Ag85 complex showed that Ag85A and Ag85B encoding plasmids induced a robust Th1-like response towards native Ag85, characterized by elevated levels of IL-2, IFNγ, and TNFα, and low levels of IL-4, IL-6, and IL-10. Cytotoxic T cell activity was generated in in vitro restimulated splenocyte cultures from Ag85A and Ag85B DNA vaccinated mice. Moreover, vaccination with Ag85A and Ag85B DNA conferred significant protection against mycobacterial replication in lungs of mice subsequently infected with live organisms [104, 105]. Protection has also been reported with DNA vaccines using genes encoding other secreted proteins like ESAT-6 and MPT64, however, DNA-Ag85B provided better protection than DNA-ESAT-6 and DNA-MPT-64 [112]. Furthermore, coimmunization with the three vectors resulted in a greater degree of protection than that induced by any single vector. This protective efficacy was associated with the emergence of IFN-γ secreting T cells earlier than in infected animals immunized with the control vector lacking mycobacterial DNA [112]. This early and increased response of IFN-γ secreting T cells may serve as a correlate of protective immunity for anti-TB vaccines [113].

In another study to compare the protective effect of single and multivalent combinantion, Morris *et al.* used single DNA vaccines encoding the antigens MPT-63 and MPT-83 of *M. tuberculosis*. These vaccines provided partial protection against an aerogenic challenge with *M. tuberculosis* in the mouse model of pulmonary TB. However, immunization with multivalent combination DNA vaccine (containing the ESAT-6, MPT-64, MPT-63, and KatG constructs) generated immune responses that indicated an absence of antigenic competition as shown by the induction of antigen-specific cell mediated and humoral responses to each component of the multivalent vaccine. More importantly, the combination vaccine induced a strong protetive effect relative to the protection generated by live BCG vaccine [106]. Among the non-secreted proteins, 38 kDa [114] and the conserved heat shock proteins, such as hsp65 [108, 115-117] have also shown protective effects as DNA vaccines in animal models of TB.

In addition to immunoprophylaxis, an ideal anti-TB vaccine should also have efficacy as an immunotherapeutic agent. This is specially required for MDR-TB patients refractory to the first line anti-TB drugs. Lowrie *et al.* used vaccination with hsp65 to extend the use of DNA

vaccination from prophylactic to therapeutic application [118]. Their work showed that mice heavily infected with *M. tuberculosis* by intravenous route could be treated with a series of four doses of plasmid DNA encoding hsp65 given intramuscularly at 2-week intervals. The number of live bacteria in spleen and lungs declined rapidly 2 months and 5 months after the first dose of DNA. Much smaller effects were obtained with plasmid expressing other mycobacterial antigens such as hsp70, or ESAT-6. A single dose of BCG failed to induce similar effects. The therapeutic effect of the DNA vaccination involved a switch from type-2 immune responses to type-1 immune response characterized by high levels of IFN-γ and very low levels of IL-4 [118]. Adoptive transfer of protection with T cell clones and in vitro tests of clone function indicated that the effects were primarily mediated by antigen specific CD8+ T cells that produced IFN-γ and killed the bacteria during granule-dependent lysis of infected macrophages [119]. However, all DNA vaccines may not be useful in immunotherapy as shown by ineffectiveness of DNA-Ag85A in mice with established infection with *M. tuberculosis* [120]. Thus each vaccine candidate has to be evaluated for its efficacy in immunotherapy.

One of the major problems in TB is reactivation disease, which may occur even when the patients are given full course of treatment and become clinically as well as bacteriologically cured. This occurs due to the existence of persisters at some hidden sites. These persisters get reactivated some time in life, most probably due to the weakening of the protective cell mediated immunity and thus lead to the reactivation disease. Studies have been conducted to determine if reactivation could be prevented in the mouse model of TB after vaccination with DNA vaccines. In experiments with DNA-hsp65, animals were injected with live *M. tuberculosis* followed by treatment with anti-TB drugs to decrease the bacterial load to undetectable level. When the mice were given immunosuppressive therapy to weaken the protective immunity, the reactivation disease occurred in the animals showing that the anti-TB treatment failed to provide an sterilizing effect. However, the reactivation was not seen in mice given DNA-hsp65 vaccine prior to immuno-suppressive therapy, thus showing that immunization with the DNA-hsp65 vaccine provided a sterilizing effect [118]. These studies suggest that some of new candidate anti-TB vaccines based on naked DNA formulations may be useful in preventing both primary TB as well as reactivation disease due to the weakening of immunological defense mechanisms some times in life. Application of such immunotherapy in conjunction with conventional chemotherapy might results in faster or more certain cure of the disease in man. Furthermore, similar vaccines used as prophylactic and therapeutic agents might be able to both prevent establishment of the persistent state and eliminate if it is already established [119].

CONCLUSIONS

By using the tools of biotechnology, considerable progress has been made during the last 15 years to identify *M. tuberculosis* antigens recognized by protective Th1 cells. More recently, comparison of genome sequence of *M. tuberculosis* with other mycobacteria has led to the identification of DNA regions specific for *M. tuberculosis*. These regions encode antigens that are promising for specific diagnosis and new vaccines against TB. Vaccination with promising recombinant antigens along with appropriate adjuvants or in the form of DNA vaccines has shown protection in animal models of TB. However, these candidate vaccines are yet to be evaluated for safety and efficacy in humans.

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